

Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide

Hugues Ouellet*[†], Yannick Ouellet*[†], Christian Richard[†], Marie Labarre[†], Beatrice Wittenberg[‡], Jonathan Wittenberg[‡], and Michel Guertin^{†§}

[†]Department of Biochemistry and Microbiology, Faculty of Sciences and Engineering, Laval University, QC, Canada G1K 7P4; and [‡]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461

Edited by Louis J. Ignarro, University of California, School of Medicine, Los Angeles, CA, and approved February 22, 2002 (received for review January 10, 2002)

Mycobacterium tuberculosis, the causative agent of human tuberculosis, and *Mycobacterium bovis* each express two genes, *glbN* and *glbO*, encoding distantly related truncated hemoglobins (trHbs), trHbN and trHbO, respectively. Here we report that disruption of *M. bovis* bacillus Calmette–Guérin *glbN* caused a dramatic reduction in the NO-consuming activity of stationary phase cells, and that activity could be restored fully by complementing knockout cells with *glbN*. Aerobic respiration of knockout cells was inhibited markedly by NO in comparison to that of wild-type cells, indicating a protective function for trHbN. TyrB10, which is highly conserved in trHbs and interacts with the bound oxygen, was found essential for NO consumption. Titration of oxygenated trHbN (trHbN-O₂) with NO resulted in stoichiometric oxidation of the protein with nitrate as the major product of the reaction. The second-order rate constant for the reaction between trHbN-O₂ and NO at 23°C was 745 μM⁻¹·s⁻¹, demonstrating that trHbN detoxifies NO 20-fold more rapidly than myoglobin. These results establish a role for a trHb and demonstrate an NO-metabolizing activity in *M. tuberculosis* or *M. bovis*. trHbN thus might play an important role in persistence of mycobacterial infection by virtue of trHbN's ability to detoxify NO.

M*ycobacterium tuberculosis* infects over 1.8 billion persons worldwide, causing 1.5 million deaths per year. Of the infected population, 90% harbor the bacterium but do not display symptoms (1). In most healthy individuals, the initial *M. tuberculosis* infection is contained by the immune system, which forces the bacteria to enter a latent state, often for several years, with possible reactivation later in life. Animal models suggest that the initial event in *Mycobacterium* infection involves entry and multiplication within inactivated macrophages. Within the macrophages the bacteria multiply in a specialized vacuolar compartment called the phagosome. After an initial rapid growth phase, infected macrophages are surrounded and walled off by newly recruited activated macrophages to form the characteristic caseous granuloma (2). Nitric oxide (NO) produced by the activated macrophages contributes strongly to induction and maintenance of bacilli dormancy (2–11). However, tuberculous infection is in dynamic balance that teeters for years in a competition between host immunity and *M. tuberculosis* growth, indicating that an endogenous mechanism for NO resistance operates in the tubercle bacillus. How *M. tuberculosis* copes with NO therefore is a key issue.

NO plays an important role in host defense against microbial pathogens by inhibiting or inactivating key enzymes such as the terminal respiratory oxidases (12–16) and the iron/sulfur protein aconitase (17, 18), which is a member of the citric acid cycle. NO also combines at near diffusion-limited rate with superoxide produced by respiring cells to form the highly oxidizing agent peroxynitrite (19, 20).

In response to the host defense, microorganisms have developed various resistance mechanisms by which toxic effects of NO can be evaded. One such mechanism is mediated by the two-domain flavohemoglobins (FHbs) that are found in certain

bacteria and yeasts. FHb detoxifies NO in a rapid reaction of the heme-bound O₂ with NO to form the innocuous nitrate and ferric FHb [Fe(II)O₂ + NO → Fe(III) + NO₃⁻] (21–26). This same function is fulfilled by animal myoglobin (Mb) and Hb (27–29).

M. tuberculosis does not possess an FHb gene (30) but does encode small Hbs, called truncated Hbs (trHbs). trHbs represent a class of small oxygen-binding hemoproteins widely distributed in bacteria, plants, and unicellular eukaryotes and forming a group separate from other Hbs including FHbs (31, 32). Three distantly related groups are distinguished within the trHb family, and some organisms express trHb encoded by genes in more than one group (31). Thus far, no functional roles have been described for trHbs, although recently *M. tuberculosis* trHbO has been shown to enhance oxygen uptake of *Escherichia coli* membrane fractions (33).

In *M. tuberculosis* and *Mycobacterium bovis* two genes, *glbN* and *glbO*, encode trHbs from group I (trHbN) and group II (trHbO), respectively (34, 35). Sequence analysis showed that *M. tuberculosis* trHbN and trHbO are identical to their *M. bovis* counterparts (31). Physiological studies performed with *M. bovis* bacillus Calmette–Guérin (BCG) have demonstrated that trHbO is expressed throughout the growth phase, but in contrast trHbN expression is enhanced greatly during the stationary phase (35). trHbN binds oxygen with great affinity ($P_{50} = 0.013$ mm Hg at 20°C) because of a fast combination (25 μM⁻¹·s⁻¹) and slow dissociation (0.2 s⁻¹) of O₂ (35). Resonance Raman studies in conjunction with site-directed mutagenesis suggested that trHbN heme iron coordination may be optimized for performing O₂/NO chemistry (36). It has been proposed that *in vivo* trHbN, by retaining bound O₂, may ensure a low but critical level of local O₂ availability necessary to afford protection against NO, assuring survival of *M. tuberculosis* in the hypoxic environment of the granuloma (35, 36). Here we report that trHbN actively detoxifies NO and protects aerobic respiration from NO inhibition.

Material and Methods

Bacterial Growth. *M. bovis* BCG (ATCC 35734) was grown in Middlebrook 7H9 (Becton Dickinson) medium supplemented with 0.6% glycerol/10% ADC (5% BSA/2% glucose/0.85% NaCl)/0.05% Tween-80 as described (35). *E. coli* XL1-Blue strain (Stratagene) was grown in Luria–Bertani medium supplemented with the appropriate antibiotic.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: FHb, flavohemoglobin; Mb, myoglobin; trHbs, truncated Hbs; BCG, bacillus Calmette–Guérin; WT, wild type.

*H.O. and Y.O. contributed equally to this work.

[§]To whom reprint requests should be addressed. E-mail: mguertin@bcm.ulaval.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Construction of a *glnB* Mutant of *M. bovis* BCG. Plasmid pMG-03 was used for the disruption of *glnB* and was constructed as follows. A 4.7-kb *Bam*HI fragment containing the entire *glnB* and *lprI* genes flanked by 0.7 and 3.0 kb of upstream and downstream sequences, respectively, was subcloned into the *Bam*HI site of pBluescript SK(−) (Stratagene) to give pMG-01 (ref. 35; Fig. 1A). The *glnB* gene was disrupted by cloning into the unique *Apa*I site a 1.2-kb cassette containing the *aph* gene for resistance to kanamycin to give pMG-02. Finally, the 5.9-kb *Bam*HI fragment of pMG-02 was subcloned into the *Bam*HI site of pPR27, a mycobacterial vector carrying a temperature-sensitive origin of replication and the *sacB* gene (37) to give pMG-03. Plasmid DNA containing the disrupted *glnB* gene was electroporated into *M. bovis* BCG cells, and transformants were selected on 7H10 plates containing 25 μ g/ml of kanamycin at 30°C. Two independent kanamycin-resistant clones were grown in 7H9 medium to mid-log and plated on 7H10 plates supplemented with ADC/0.5% glycerol/25 μ g/ml kanamycin/2% sucrose at 39°C. After 8 weeks, transformants were picked and analyzed by Southern hybridization.

Complementation of the *glnB* Mutant. A 1.4-kb PCR fragment carrying *glnB* and *lprI* coding sequences as well as 125 and 208 bp of 5'- and 3'-flanking sequences was amplified from *M. bovis* BCG (ATCC 35734) genomic DNA by using the forward primer 5'-CAGGTACCCGACCAAGGATCTGTTTGC-3' and reverse primer 5'-TAGGTACCGTTGCATACCCATGTTGC-3'. Both primers contain a *Kpn*I restriction site. The amplicon was digested with *Kpn*I and subcloned into the vector pBluescript to give pMG-11. A second plasmid, pMG-12, containing a frame-shift mutation in the *lprI* gene, was constructed from pMG-11 by introducing an 8-bp *Bcl*I linker at the *Stu*I of *lprI*. The tyrosine found at the position 33 (B10) in trHbN was changed to a phenylalanine by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) into pMG-11 to give pMG-13. These three plasmids were sequenced on both strands using local sequencing facilities. The three 1.4-kb fragments containing the *glnB-lprI* genes then were subcloned into pMV206/hyg (38) bearing the *hyg* gene for resistance to hygromycin to give pMG-14, pMG-15, and pMG-16. These constructs were electroporated into Δ HbN cells, and transformants were isolated at 37°C on 7H10 plates containing 50 μ g/ml hygromycin.

RNA Isolation and Reverse Transcription-PCR. Total cellular RNA was isolated from *M. bovis* BCG (ATCC 35734) cells harvested 24 h after the beginning of the stationary phase by using an RNeasy kit (Qiagen, Chatsworth, CA). First-strand synthesis was performed with Omniscript reverse transcriptase (Qiagen) and a reverse primer (5'-AGCCGGTGGTCCAGACTG-3') located within the coding sequence of *lprI* gene according to the manufacturer's protocol. PCR amplification was performed by using *Taq* DNA polymerase (GIBCO/BRL, Life Technologies, Burlington, ON, Canada) and a set of primers (forward, 5'-GGAAATCCTCGGCGTCAT-3', and reverse, 5'-AGCCGGTGGTCCAGACTG-3') located in *glnB* and *lprI* genes, respectively.

Preparation of NO-Saturated Solutions. NO solutions were prepared by first deoxygenating water or a 50 mM phosphate buffer (pH 7.5) containing 50 μ M EDTA for at least 30 min with N₂ and then saturating them with NO. The final NO concentration (1.5–2.0 mM) was measured with an NO electrode (World Precision Instruments, Sarasota, FL). Diluted NO solutions were prepared in 50 mM phosphate buffer (pH 7.5) containing 50 μ M EDTA inside an MBraun Labmaster 100 glovebox using gas-tight Sample-Luer Lock Hamilton syringes. Hamilton gas-tight syringes were used to withdraw and inject NO solutions.

Determination of NO Consumption and Respiration Rates. NO and O₂ consumption was measured in a thermostated 2-ml reaction chamber (World Precision Instruments) with ISO-NO and ISO2 electrodes (World Precision Instruments), respectively. The NO and O₂ electrodes were calibrated according to the manufacturer's recommendations. Cells were harvested after 24 h in stationary phase, centrifuged at 3,000 \times g at 4°C for 15 min and resuspended at either 1.0 or 4.0 OD_{580 nm} (1 A₅₈₀ \equiv 1 \times 10⁸ cells per ml) in PBS buffer containing 0.05% (vol/vol) Tween-80. NO consumption was measured after the addition of NO to the cell suspension (2 ml) at 37°C. We tested the effect of adding glucose to the cell suspension and found no difference in either NO or O₂ consumption.

Titration of trHbN·O₂ with NO. trHbN was purified as described (35). trHbN·O₂ samples were prepared by exposing to air the ligand-free ferrous trHbN prepared anaerobically. NO was added sequentially from a saturated stock solution (1.5–2.0 mM) with a gas-tight Hamilton syringe into a 1.5-ml quartz cuvette sealed with a rubber septum and containing 1.4 ml of 15 μ M trHbN·O₂ in 50 mM phosphate buffer (pH 7.5) containing 50 μ M EDTA. Spectra were recorded at 23°C with a Cary 3E spectrophotometer until equilibrium was achieved.

Determination of Nitrite and Nitrate Formed in the Reaction of trHbN·O₂ with NO. All solutions were prepared by using ultra pure water (Fluka) containing less than 0.000001% nitrate). trHbN·O₂ and oxygenated horse heart Mb (Mb·O₂, Sigma-Aldrich) were prepared at a concentration of 25 μ M as described above. NO was added at a final concentration of 25 μ M from a saturated solution (1.5–2.0 mM). Protein samples were denatured by heat (100°C) for 5 min and centrifuged at 13,000 \times g for 5 min. The supernatants were assayed for the nitrite and nitrate content by using the Griess reagent and vanadium(III) (39). We measured no nitrate and 0–0.2 μ M nitrite in the saturated NO solutions.

Stopped-Flow Kinetic Analysis. Kinetic studies were carried out at 23°C with an Applied Photophysics SX.18MV single-wavelength stopped-flow spectrophotometer equipped with a photodiode array detector. The optical path length was 1 cm. The dead time of the apparatus was 0.9 ms. Solutions of Mb·O₂ and trHbN·O₂ (1–5 μ M) were prepared in 50 mM phosphate buffer (pH 7.5) containing 50 μ M EDTA. Protein concentrations were determined by measuring the absorbance at 417 and 542 nm (ϵ_{417} = 128 mM⁻¹·cm⁻¹, ϵ_{542} = 13.9 mM⁻¹·cm⁻¹) for Mb·O₂ and 416 and 545 nm (ϵ_{416} = 94.4 mM⁻¹·cm⁻¹, ϵ_{545} = 10.0 mM⁻¹·cm⁻¹) for trHbN·O₂. Kinetic traces (averages of at least five single traces of three separate experiments) taken at 409 (horse heart Mb) and 404 nm (trHbN) were analyzed with the SX.18MV operating software.

Results

Organization of the *glnB* Locus. Sequence analysis of the *M. bovis* and *M. tuberculosis* genomes revealed that the genomic organization of the *glnB* locus is conserved (Fig. 1A; refs. 34 and 35). It also indicated the presence of a second gene (*lprI*), the initiation codon of which was separated from the stop codon of *glnB* by only 58 nucleotides, suggesting that both genes were cotranscribed. To verify cotranscription of both genes, total RNA was isolated from *M. bovis* BCG cells harvested 24 h after the beginning of the stationary phase and used to perform reverse transcription-PCR with a pair of primers that allows coamplification of *glnB* and *lprI*. A single product of the expected size was detected after electrophoresis on agarose gel, indicating that both genes are cotranscribed *in vivo* (data not shown).

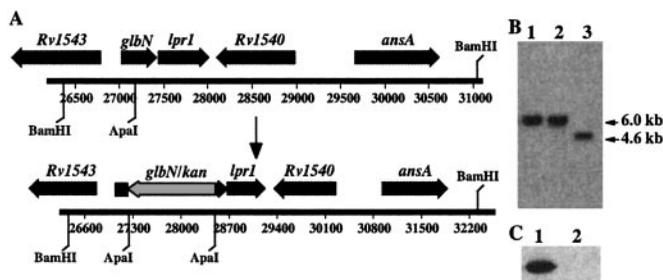


Fig. 1. Inactivation of *glbN* from *M. bovis* BCG (ATCC 35734). (A) Schematic representation of the *glbN* locus in *M. bovis* before and after the insertion of the *kan* resistance gene. The same organization is observed in *M. tuberculosis*. (B) Southern blot of *Bam*HI-digested genomic DNA showing the *glbN*-containing fragment of WT BCG (lane 3, expected size of 4,600 bp) and the hybridizing fragment of two *glbN* mutants (lanes 1 and 2, expected size of 5,800 bp). The fragment size of the mutants is consistent with the insertion of the 1.2-kb *kan* resistance gene. (C) Western blot analysis of total proteins (10.0 μ g) from WT BCG (lane 1) and a Δ HbN mutant (lane 2) harvested 24 h after entry into stationary phase. The detection was performed by using polyclonal antibodies raised against trHbN.

Gene Disruption of *glbN* in *M. bovis* BCG. To inactivate *glbN*, a 1.2-kb *aph* gene (kanamycin resistance) was inserted at its unique *Apa*I site (Fig. 1A). The *Bam*HI fragment containing disrupted *glbN* was cloned in the delivering vector pPR27 and used to transform *M. bovis* BCG cells (Fig. 1A). This plasmid allows a gene replacement strategy that uses the counterselective properties of the *sacB* gene and a mycobacterial thermosensitive (*ts*) origin of replication (37). The presence of the *ts* origin of replication in conjunction with *sacB* facilitates the selection of mutants. Kanamycin-resistant transformants that grew at 39°C on 7H10/kanamycin-containing 2% sucrose plates were conserved for Southern blot analysis. As expected, for allelic exchange mutants, clones were found that presented a single hybridizing fragment 1.2 kb longer than in the wild-type (WT) strain (Fig. 1B). This 1.2-kb increase corresponded to the size of the *aph* gene that was inserted into the mutated allele. The selected *glbN* mutant thereafter was designated Δ HbN. Western blot analysis confirmed that Δ HbN cells no longer express trHbN (Fig. 1C).

trHbN Is Not Essential for Cell Growth. Growth characteristics of Δ HbN cells were compared with those of its parental strain. No difference in colony morphology was visible when the strains were plated on solid medium. When grown in liquid medium with shaking, no difference was observed in the growth curves of the mutant versus the WT strains at 30 or 37°C. No significant difference in survival was noted between the two strains throughout the growth phase. These results suggest that trHbN is not essential at least under laboratory growth conditions.

trHbN Is Critical for NO Metabolism *in Vivo*. To determine whether trHbN is necessary for NO metabolism, we examined NO consumption by stationary phase cells of WT and Δ HbN strains. As shown in Fig. 2A, WT cells readily consumed NO in comparison to Δ HbN cells, the latter displaying only activity that was quite similar to buffer alone. Although these results strongly suggested that trHbN was responsible for the NO-metabolizing activity, it still remained possible that *lpr1*, not *glbN*, was metabolizing NO. Indeed, insertion of the *kan* gene in *glbN* may have interfered with the transcriptional or translational machinery and abolished synthesis of the LprI protein. To verify this possibility, we transformed Δ HbN cells with the replicative plasmid pMG-12 bearing *glbN* and inactivated *lpr1*. As shown in Fig. 2A, NO-consuming activity was restored fully in complemented Δ HbN cells (Δ HbN:HbN). In fact, the activity was even

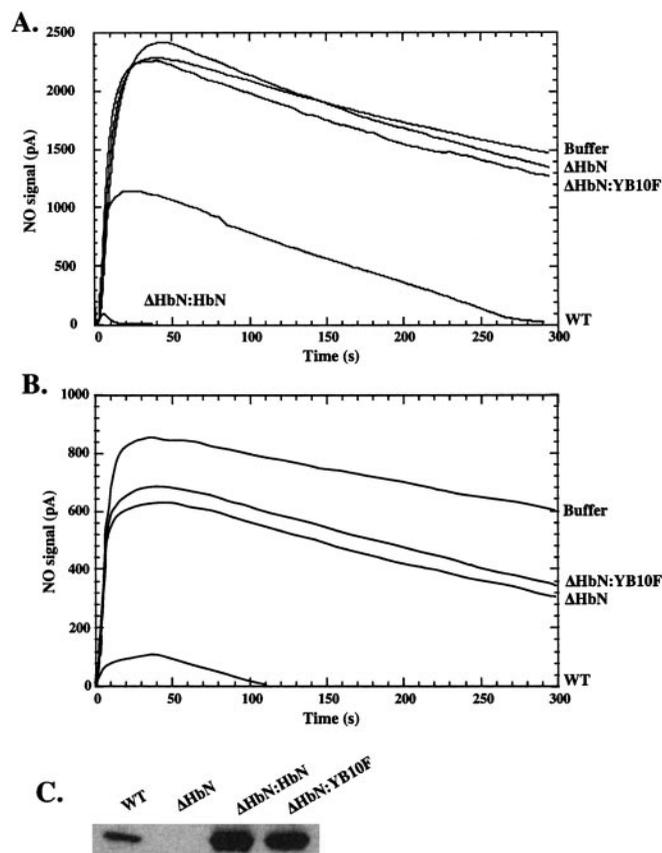


Fig. 2. trHbN is required for aerobic metabolism of NO in *M. bovis* BCG (ATCC 35734). NO (A, 1.0 μ M; B, 0.5 μ M) was added to buffer or cell suspensions. NO was monitored with an NO electrode. (C) Aliquots of cell suspensions were taken for Western blot analysis of soluble proteins by using the polyclonal antibodies raised against trHbN.

greater than in WT cells (Fig. 2A) because of elevated levels of trHbN (Fig. 2C). Identical results were obtained when the Δ HbN strain was transformed with plasmid pMG-11 bearing WT *glbN* and *lpr1* (data not shown). These results thus demonstrate that trHbN and not LprI metabolizes NO. Interestingly, as shown in Fig. 2B, when NO concentration was lowered below 1 μ M, an additional NO-consuming activity was detected reproducibly in Δ HbN cells. NO concentration used in Fig. 2A (1 μ M) exceeded that of trHbN in the reaction chamber (20–40 nM), indicating that NO scavenging by trHbN is not restricted to a single catalytic cycle.

Tyr10 Is Necessary for NO Detoxification by trHbN. The role of the highly conserved B10Tyr residue in regulating the NO-consuming activity of trHbN was examined. trHbN has a unique distal heme pocket structure that allows strong stabilization of the bound oxygen molecule ($k_{off} = 0.2 \text{ s}^{-1}$), largely by hydrogen bonding to the tyrosine residue at the B10 position (35, 36). Mutation of the B10Tyr residue to phenylalanine increased the oxygen dissociation rate 150-fold, indicating the importance of the tyrosine hydroxyl for tight oxygen binding (35). Fig. 2A and B shows that when plasmid pMG-13 bearing *glbN* with the B10Tyr \rightarrow Phe mutation was transformed into Δ HbN cells (Δ HbN:YB10F), the NO-consuming activity was not enhanced, which demonstrates that the B10Tyr hydroxyl is essential for the NO-detoxification by trHbN. Western blot analysis confirmed that failure to restore NO activity could not be attributed to a

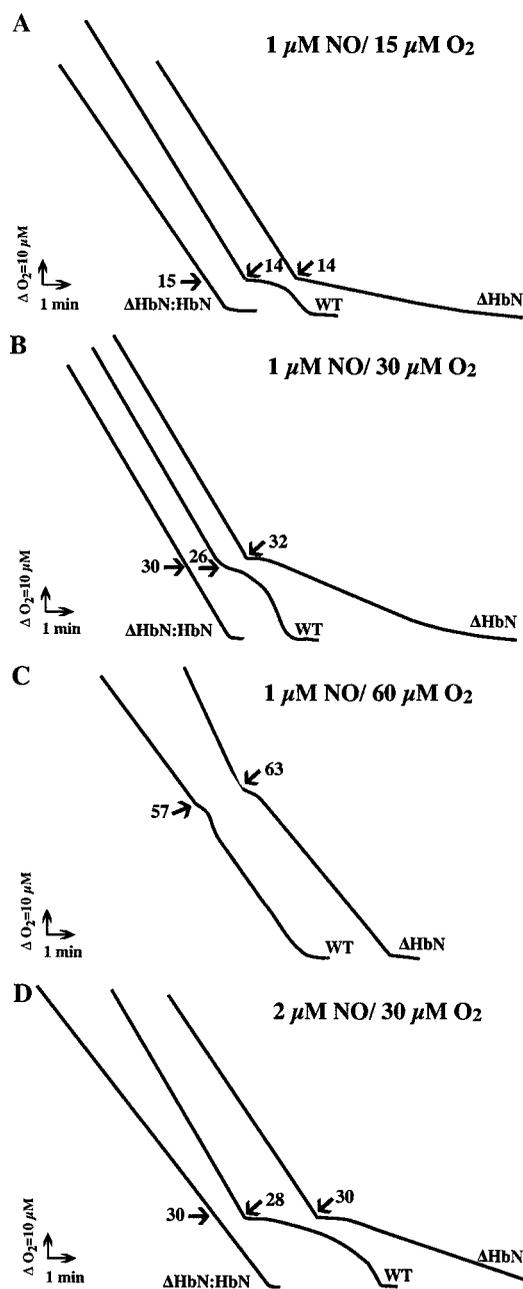


Fig. 3. trHbN affords protection for *M. bovis* BCG (ATCC 35734) respiration from NO. Respiration of washed cell suspensions was measured with an oxygen electrode apparatus. A solution of NO was added at the times indicated by the arrows to give a final concentration of 1 (A–C) and 2 (D) μM NO. The O_2 concentration at the times of injection is shown alongside each addition. The experiments were repeated at least three times with similar results. The duration of inhibition was determined as the period between the addition of NO and the point obtained by extrapolation of the residual respiration rate (after inhibition was relieved) and the inhibited rate.

reduced expression or instability of the mutant protein in $\Delta\text{HbN}:\text{YB10F}$ cells (Fig. 2C).

trHbN Protects Aerobic Respiration in Stationary Phase Cells of *M. bovis* BCG. To understand better the metabolic function of trHbN, we determined the sensitivity of WT and ΔHbN cell respiration to NO under various O_2 and NO concentrations. In the absence of NO, the respiration rates of WT, ΔHbN , and complemented

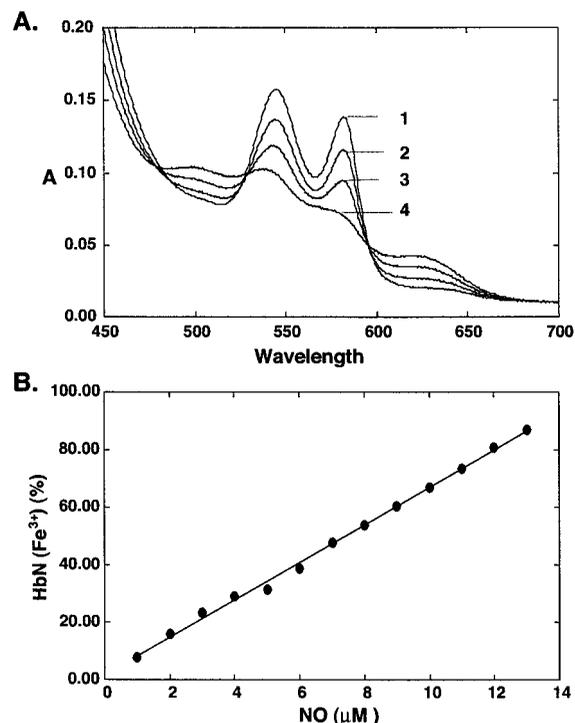


Fig. 4. Titration of NO against trHbN- O_2 . Repeated additions of a saturated aqueous NO solution were made to trHbN- O_2 (15 μM heme content). (A) Spectra were recorded after equilibrium was reached. The addition of NO led to the rapid appearance of a partial oxidized spectrum. A spectrum of the fully oxidized proteins was obtained after 14 additions of NO (14 μM NO). Subsequent additions of NO resulted in the formation of the ferric–NO complex of the proteins (data not shown). 1, trHbN- O_2 ; 2, 3, and 4, 8, and 12 μM NO, respectively. (B) Fraction (%) of oxidized protein was plotted against NO concentration.

cells were not significantly different, indicating that trHbN does not contribute significantly to O_2 uptake. NO is known to inhibit reversibly mitochondrial and bacterial respiration by competing with O_2 for the active sites of the terminal oxidases. For these experiments, cell suspensions were allowed to consume O_2 until the desired O_2 concentration was reached. At that point, NO was injected and O_2 consumption was measured. The addition of NO to a final concentration of 1 μM in a respiring WT cell suspension in which the O_2 tension was $\approx 15 \mu\text{M}$ caused immediate inhibition of respiration (Fig. 3A). After ≈ 2 min, respiration resumed at its initial rate and proceeded until O_2 was depleted. In contrast, inhibition elicited by 1 μM NO was much greater in ΔHbN cells, and respiration never returned to the initial rate (Fig. 3A). When NO was added at progressively higher dissolved O_2 tensions, the extent and duration of inhibition was decreased in both WT and ΔHbN cells (Fig. 3B and C). On the other hand, raising the NO concentration (Fig. 3D) increased the duration of inhibition. The inhibitory effect of NO was reversed when ΔHbN cells were complemented with plasmid pMG-12 ($\Delta\text{HbN}:\text{HbN}$), which restores expression of trHbN (Fig. 3A–D). Taken together, these results demonstrate that NO reversibly inhibits aerobic respiration by quiescent BCG cells, and trHbN protects respiration from NO inhibition under biologically relevant O_2 concentrations.

trHbN Oxidizes NO to Nitrate. Several Hbs have been shown to protect cells against nitrosative stress by converting NO into nitrate in the presence of oxygen. As shown in Fig. 4, titration of trHbN- O_2 with NO *in vitro* resulted in immediate stoichiometric oxidation of the protein. Nitrate, the principal product of the

Table 1. Stoichiometric formation of nitrate in the reaction of NO with *M. bovis* BCG trHbN-O₂

Protein	NO ₂ ⁻ , μM	NO ₃ ⁻ , μM
Mb-O ₂ (25 μM)	0.2 ± 0.1	25.8 ± 0.32
TrHbN-O ₂ (25 μM)	0.2 ± 0.2	26.0 ± 0.25

Horse heart Mb-O₂ serves as a control. Aqueous NO (to a final concentration of 25 μM) was added to a solution of either trHbN-O₂ or Mb-O₂ (25 μM heme content). Spectra were recorded after equilibrium was reached to ensure that the reaction was completed. Final concentrations of nitrite and nitrate were determined as described in *Material and Methods*.

reaction, was formed in exact molar equivalence with the trHbN-O₂ consumed (Table 1).

Time Courses and Rate Constant for the Oxidation of trHbN-O₂. The NO-induced reactions of trHbN-O₂ and horse heart Mb-O₂ were studied by stopped-flow spectrophotometry. Because accurate determination of NO concentration is difficult to achieve, the proteins were present in a 10-fold excess over NO to achieve reaction kinetics that were pseudo first-order with respect to trHbN-O₂ or Mb-O₂. Fig. 5A shows the time courses of the reactions of trHbN-O₂ at pH 7.5 and 23°C (average of six traces). Kinetic traces measured at 409 and 404 nm resulted in the rapid appearance of aquomet form of the Mb and trHbN, respectively, without any evidence for intermediate(s). trHbN-O₂ and Mb-O₂ showed time courses that can be fitted to a single exponential expression. Plots of the pseudo first-order rate constants, k_{obs} , versus [trHbN-O₂] or [Mb-O₂] were linear with a y intercept ≈ 0 (Fig. 5B). The second-order rate constants, obtained from the linear plots, were 46.1 and 745 μM⁻¹s⁻¹ for the NO-mediated

oxidation of Mb-O₂ and trHbN-O₂, respectively, showing that *in vitro* trHbN-O₂ detoxifies NO ≈20-fold more rapidly than Mb-O₂. The rate constant measured for Mb is in good agreement with those reported previously (40, 41).

Discussion

In this study we demonstrate that *M. bovis* trHbN, *in situ* in living cells, rapidly removes NO and protects aerobic respiration from NO inhibition under biologically relevant O₂ tensions. By metabolizing NO to the innocuous product nitrate, trHbN also may prevent NO-mediated modification of thiol and metal centers of other critical enzymes and regulatory proteins (3, 42). In addition, it may reduce the formation of the highly reactive peroxynitrite, thereby preventing the irreversible oxidation and nitration of proteins. trHbN thus might be important to the bacilli under conditions where O₂ supply is limited and NO concentration is elevated such as is anticipated in the caseous granuloma or during the reactivation of the bacilli.

Significant NO-metabolizing activity was detected in resting *M. bovis* BCG cells not synthesizing trHbN (Fig. 2B). A likely candidate for this additional activity is trHbO, the other trHb in *M. bovis* and *M. tuberculosis*. trHbO is expressed throughout the growth period and also readily converts NO into nitrate (unpublished results).

We observed no increase in trHbN protein levels with NO-donor treatments [nitrite, 2,2'-(hydroxynitrosohydrazono) bisethanimine and *S*-nitroso-*N*-acetylpenicillamine] in logarithmic or stationary phase under aerobic conditions (unpublished results). Chemicals that impose an oxidative stress such as peroxide and menadione were also without effect on trHbN expression. These results may indicate the ubiquitous presence of NO in the natural environment of *M. bovis* and/or that trHbN function goes beyond NO detoxification as exemplified by Mb, which both scavenges NO and transports oxygen. trHbN may constitute a first line of defense against NO, allowing cells to adjust adequately to a nitrosative stress.

The second-order rate constant ($K = 745 \mu\text{M}^{-1}\text{s}^{-1}$) measured for the NO-mediated oxidation of trHbN-O₂ at 23°C and pH 7.5 is close to that of *E. coli* Fhb-O₂ (850 μM⁻¹s⁻¹; ref. 23) and is 20-fold greater than that of reaction of NO with Mb-O₂ (35–45 μM⁻¹s⁻¹; refs. 40 and 41). Based on their resonance Raman studies, Yeh *et al.* (36, 43) have postulated that the active site structure of trHbN and *E. coli* Fhb is tailored to promote O₂/NO chemistry instead of O₂ delivery, which could explain their high activity with respect to Mb. In both of these bacterial Hbs, B10Tyr strongly interacts with the bound O₂ and may provide a strong electronic pull for the O—O bond activation, just as in peroxidases. In line with these observations, we found that the B10Tyr is necessary for the reaction of trHbN-O₂ with NO. Similarly, Gardner *et al.* (23) demonstrated that changing the B10Tyr in *E. coli* Fhb for a Phe decreases the reaction rate by a factor of ≈30-fold while increasing the O₂ dissociation rate constant 80-fold.

Dioxygenation of NO by Mb-O₂ does not require hydrogen bonding between E7His and the bound O₂ (41). In fact, substitution of the E7His by amino acids, which do not form hydrogen bonds to the bound O₂, accelerated NO-mediated oxidation of Mb (41). It has been proposed that this hydrogen bond must be disrupted to allow for the entry of NO in the distal heme pocket, the process that is considered to be the rate-determining step for this reaction. In trHbN, as in other trHbs studied, x-ray (44, 45) and resonance Raman spectroscopy (31, 35, 36) indicated a very crowded pocket in which solvent access through the E7 gate is impaired totally. However, a route for ligand diffusion to and from the heme is present as a hydrophobic tunnel or cavity network through the protein matrix (31, 44, 45). Such a guiding tunnel may allow efficient diffusion of ligands to the active site. It also may accelerate the NO detoxification reaction by tran-

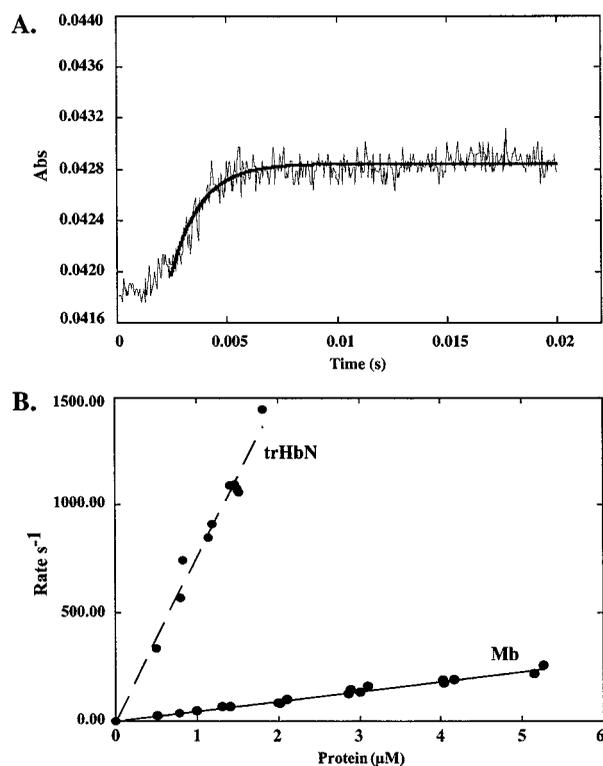


Fig. 5. Time course and rate constant for the oxidation of trHbN-O₂. (A) Time course (average of six traces) measured at 404 nm for the reaction of 0.8 μM trHbN-O₂ with ≈0.08 μM NO at pH 7.5 and 23°C. The solid line corresponds to the best fit to the data with one exponential. (B) Plots of the rates (s⁻¹) versus trHbN-O₂ and horse heart Mb-O₂ concentrations at pH 7.5 and 23°C.

siently storing several reactant NO molecules. The presence of a large and unobstructed distal heme pocket in the crystal structures of *Alcaligenes* FHB (46) and *Vitreoscilla* Hb (47) suggests that diffusion of ligand may also not be a rate-determining step in FHbs.

The conservation of the B10Tyr in trHbs, and possibly the tunnel, points to a similar function for other trHbs. It will be important to determine whether all trHbs show significant NO-scavenging activity and whether some catalyze novel reactions with specialized functions. It is noteworthy that the B10Tyr

is also highly conserved in FHbs (48). Such conservation indicates a very ancient origin for the B10Tyr as well as a conserved function. Apart from a few exceptions, B10Tyr is absent in almost all modern animal and plant Hbs. The loss of the B10Tyr and therefore of a less reactive heme-bound O₂ may have been mandatory to evolve a protein capable of transport and diffusion of O₂.

This work was supported by Natural Sciences and Engineering Research Council Grant 46306-01 (to M.G.).

1. Styblo, K. (1980) *Adv. Tuberc. Res.* **20**, 1–63.
2. Flynn, J. L. & Chan, J. (2001) *Annu. Rev. Immunol.* **19**, 93–129.
3. Stamler, J. S., Lamas, S. & Fang, F. C. (2001) *Cell* **106**, 675–683.
4. Chan, E. D., Morris, K. R., Belisle, J. T., Hill, P., Remigio, L. K., Brennan, P. J. & Riches, D. W. (2001) *Infect. Immun.* **69**, 2001–2010.
5. Chan, E. D., Chan, J. & Schluger, N. W. (2001) *Am. J. Respir. Cell Mol. Biol.* **25**, 606–612.
6. MacMicking, J. D., North, R. J., LaCourse, R., Mudgett, J. S., Shah, S. K. & Nathan, C. F. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5243–5248.
7. Chan, J., Tanaka, K., Carroll, D., Flynn, J. & Bloom, B. R. (1995) *Infect. Immun.* **63**, 736–740.
8. Chan, J., Xing, Y., Magliozzo, R. S. & Bloom, B. R. (1992) *J. Exp. Med.* **175**, 1111–1122.
9. Nathan, C. & Shiloh, M. U. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 8841–8848.
10. Shiloh, M. U. & Nathan, C. F. (2000) *Curr. Opin. Microbiol.* **3**, 35–42.
11. Garcia, I., Guler, R., Vesin, D., Olleros, M. L., Vassalli, P., Chvatchko, Y., Jacobs, M. & Ryffel, B. (2000) *Lab. Invest.* **80**, 1385–1397.
12. Brunori, M. (2001) *Trends Biochem. Sci.* **26**, 21–23.
13. Brunori, M., Giuffrè, A., Sarti, P., Stubauer, G. & Wilson, M. T. (1999) *Cell. Mol. Life Sci.* **56**, 549–557.
14. Stevanin, T. M., Ioannidis, N., Mills, C. E., Kim, S. O., Hughes, M. N. & Poole, R. K. (2000) *J. Biol. Chem.* **275**, 35868–35875.
15. Cleeter, M. W., Cooper, J. M., Darley-Usmar, V. M., Moncada, S. & Schapira, A. H. (1994) *FEBS Lett.* **345**, 50–54.
16. Brown, G. C. & Cooper, C. E. (1994) *FEBS Lett.* **356**, 295–298.
17. Gardner, P. R., Costantino, G. & Salzman, A. L. (1998) *J. Biol. Chem.* **273**, 26528–26533.
18. Gardner, P. R., Costantino, G., Szabo, C. & Salzman, A. L. (1997) *J. Biol. Chem.* **272**, 25071–25076.
19. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G. & Koppenol, W. H. (1997) *Chem. Res. Toxicol.* **10**, 1285–1292.
20. Pfeiffer, S., Gorren, A. C., Schmidt, K., Werner, E. R., Hansert, B., Bohle, D. S. & Mayer, B. (1997) *J. Biol. Chem.* **272**, 3465–3470.
21. Gardner, P. R., Gardner, A. M., Martin, L. A. & Salzman, A. L. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 10378–10383.
22. Hausladen, A., Gow, A. J. & Stamler, J. S. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14100–14105.
23. Gardner, A. M., Martin, L. A., Gardner, P. R., Dou, Y. & Olson, J. S. (2000) *J. Biol. Chem.* **275**, 12581–12589.
24. Gardner, P. R., Gardner, A. M., Martin, L. A., Dou, Y., Li, T., Olson, J. S., Zhu, H. & Riggs, A. F. (2000) *J. Biol. Chem.* **275**, 31581–31587.
25. Hausladen, A., Gow, A. & Stamler, J. S. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 10108–10112.
26. Liu, L., Zeng, M., Hausladen, A., Heitman, J. & Stamler, J. S. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 4672–4676.
27. Fogel, U., Merx, M. W., Godecke, A., Decking, U. K. & Schrader, J. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 735–740.
28. Wennmalm, A., Benthin, G. & Petersson, A. S. (1992) *Br. J. Pharmacol.* **106**, 507–508.
29. Minning, D. M., Gow, A. J., Bonaventura, J., Braun, R., Dewhirst, M., Goldberg, D. E. & Stamler, J. S. (1999) *Nature (London)* **401**, 497–502.
30. Poole, R. K. & Hughes, M. N. (2000) *Mol. Microbiol.* **36**, 775–783.
31. Wittenberg, J. B., Bolognesi, M., Wittenberg, B. A. & Guertin, M. (2002) *J. Biol. Chem.* **277**, 871–874.
32. Moens, L., Vanfleteren, J., Van de Peer, Y., Peeters, K., Kapp, O., Czeluzniak, J., Goodman, M., Blaxter, M. & Vinogradov, S. (1996) *Mol. Biol. Evol.* **13**, 324–333.
33. Pathania, R., Navani, N. K., Rajamohan, G. & Dikshit, K. L. (Jan. 16, 2002) *J. Biol. Chem.*, 10.1074/jbc.M111478200.
34. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, et al. (1998) *Nature (London)* **393**, 537–544.
35. Couture, M., Yeh, S. R., Wittenberg, B. A., Wittenberg, J. B., Ouellet, Y., Rousseau, D. L. & Guertin, M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 11223–11228.
36. Yeh, S. R., Couture, M., Ouellet, Y., Guertin, M. & Rousseau, D. L. (2000) *J. Biol. Chem.* **275**, 1679–1684.
37. Pelicic, V., Jackson, M., Reytrat, J. M., Jacobs, W. R., Jr., Gicquel, B. & Guilhot, C. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 10955–10960.
38. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H., Hatfull, G. F., et al. (1991) *Nature (London)* **351**, 456–460.
39. Miranda, K. M., Espey, M. G. & Wink, D. A. (2001) *Nitric Oxide* **5**, 62–71.
40. Herold, S., Exner, M. & Nauser, T. (2001) *Biochemistry* **40**, 3385–3395.
41. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr. & Olson, J. S. (1996) *Biochemistry* **35**, 6976–6983.
42. Gardner, P. R. (1997) *Biosci. Rep.* **17**, 33–42.
43. Mukai, M., Mills, C. E., Poole, R. K. & Yeh, S. R. (2001) *J. Biol. Chem.* **276**, 7272–7277.
44. Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., Moens, L. & Bolognesi, M. (2000) *EMBO J.* **19**, 2424–2434.
45. Milani, M., Pesce, A., Ouellet, Y., Ascenzi, P., Guertin, M. & Bolognesi, M. (2001) *EMBO J.* **20**, 3902–3909.
46. Ermler, U., Siddiqui, R. A., Cramm, R. & Friedrich, B. (1995) *EMBO J.* **14**, 6067–6077.
47. Tarricone, C., Galizzi, A., Coda, A., Ascenzi, P. & Bolognesi, M. (1997) *Structure (London)* **5**, 497–507.
48. Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C. & Ascenzi, P. (1997) *Prog. Biophys. Mol. Biol.* **68**, 29–68.